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SHORT COMMUNICATION

Gravity-induced reorientation of cortical microtubules observed *in vivo*

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Summary

Cortical microtubules play an important role during morphogenesis by determining the direction of cellulose deposition. Although many triggers are known that can induce the reorientation of cortical plant microtubules, the reorientation mechanism has remained obscure. In our approach, we used gravitropic stimulation which is a strong trigger for microtubule reorientation in epidermal cells of maize coleoptiles. To visualize the gravitropically induced microtubule reorientation in living cells, we injected rhodamine-conjugated tubulin into epidermal cells of intact maize coleoptiles that were exposed to gravitropic stimulation. From these *in vivo* observations, we propose a reorientation mechanism consisting of four different stages: (1) a transitional stage with randomly organized microtubules; (2) emergence of a few microtubules in a slightly oblique orientation; (3) co-alignment: neighbouring microtubules adopt the oblique orientation resulting in parallel organized microtubules; and (4) the angle of these parallel, organized microtubules increases gradually. Thus, the overall reorientation process could include selective stabilization/disassembly of microtubules (stage 2) as well as movement of individual microtubules (stages 3 and 4).

Introduction

Cortical microtubules, cytoskeletal components that are unique to plants, play an important role in plant morphogenesis, being involved in the control of cell growth and cell shape. They are thought to regulate the shape of plant cells by directing the movement of cellulose synthase complexes in the plasma membrane (Giddings and Staehelin, 1991). The way in which the cellulose microfibrils are laid down determines the direction of cell expansion. Cortical microtubules can reorient in response to various signals, such as hormones, gravity, light or physical stimuli (reviewed in Nick, 1998). However, the mechanism of reorientation has for a long time remained unclear due to limitations of the immunofluorescence technique, based on fixed material. Microinjection of fluorescence-labelled tubulin into living cells has made it possible to observe microtubules for up to several hours (Wasteneys *et al.*, 1993; Wymer *et al.*, 1997; Yuan *et al.*, 1994; Zhang *et al.*, 1990). This technique allowed, for the first time, the demonstration that cortical microtubules are highly dynamic. For instance, studies on fluorescence recovery after photobleaching revealed a half time for cortical plant microtubules of only 60–67 sec compared to reported half times for animal cells of over 200 sec (Hush *et al.*, 1994). Furthermore, microinjection of rhodamine–tubulin into pea epidermal cells showed that the spontaneous reorientation of microtubules from transverse to longitudinal occurred in as little as 40 min (Yuan *et al.*, 1994). First, 'discordant' microtubules appeared in the new direction then the array went through a mixed phase before parallelism was restored in the new direction. By adding gibberellic acid, Lloyd *et al.* (1996) were able to see the reverse transition, from longitudinal to transverse, in microinjected cells. This raises the question of how general is the mechanism by which microtubules alternate between transverse and longitudinal orientations. To answer this and to learn more about the details of the reorientation process we have examined microtubule reorientation in maize coleoptiles in response to gravitropic stimulation, a non-invasive trigger. Caused by a shift of auxin across the coleoptile, microtubules on the faster growing lower flank of the coleoptile are transverse, while microtubules on the slower growing upper flank assume a longitudinal orientation with respect to the long axis of the cell (Nick *et al.*, 1990). Thus, by shifting the orientation in which microfibrils are laid down, the dynamic microtubule template (which aligns differently on opposite flanks of the coleoptile) is believed to control the gravitropic bending.

In this study, we followed the response of individual cells in unfixed coleoptiles injected with rhodamine-labelled tubulin. Our observations indicate that microtubules, in response to the gravitropic stimulus, reorient from transverse to longitudinal by passing a mixed phase

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before parallelism is restored, thus making the angle gradually steeper.

Results

Gravity is the trigger

Microtubules are known to change their orientation in response to gravitropic stimulation (Nick *et al.*, 1990). In epidermal cells of maize coleoptiles, microtubules on the upper coleoptile side reorient from transverse to longitudinal whereas they adopt a transverse orientation at the lower side. To monitor this microtubule reorientation on the upper side from transverse to longitudinal continuously in living cells after microinjection of rhodamine-tubulin, coleoptiles were glued to microscope slides. Immunofluorescence studies (data not shown) confirmed that the tissue attachment did not impair the microtubule response to gravity: after 1 h of gravitropic stimulation more than 75% of the microtubules in the upper flank were steeply oblique or longitudinal. In principle, the attachment of the coleoptiles could produce a mechanical stress which, in itself, could trigger microtubule reorientation (Fischer and Schopfer, 1997). To control for this possibility, coleoptiles were prepared exactly as for microinjection, except that they were kept in a vertical position, i.e. without gravitropic stimulation. Attachment of the coleoptile to the glass reduced growth to 15–25% of the control, but had almost no effect on microtubule orientation (data not shown). Thus, it is gravity and not the attachment to the slide that triggers microtubule reorientation.

Gravity-induced microtubule reorientation in vivo

To follow this gravi-stimulated microtubule reorientation *in vivo*, rhodamine-conjugated neurotubulin was microinjected into epidermal cells of freshly isolated, intact maize coleoptiles. For gravitropic stimulation, the coleoptiles were kept in a horizontal position. Labelled tubulin became incorporated into microtubules within a few minutes after injection. In many cells, the gravitropic stimulus triggered the reorientation of microtubules from initially more-or-less transverse arrays to steeply oblique/longitudinal arrays (Figures 1 and 2). This reorientation process could be subdivided into three stages: (1) a transitional period about 30 min after the onset of stimulation during which longitudinal microtubules coexisted with microtubules that were oriented in the original transverse orientation resulting in a patchwork of different orientations within the same cell (Figures 1b and 2a). (2) From this random transitional state, a new order gradually emerged with parallel bundles of microtubules in oblique orientation (Figures 1c,d and 2b). This alignment process seemed to start in one area, first with coalignment between neighbouring areas which then proceeded throughout the cell (Figure 1c,d). (3) Once the microtubules were aligned in these parallel arrays, the angle increased smoothly over time (Figure 1c–f).

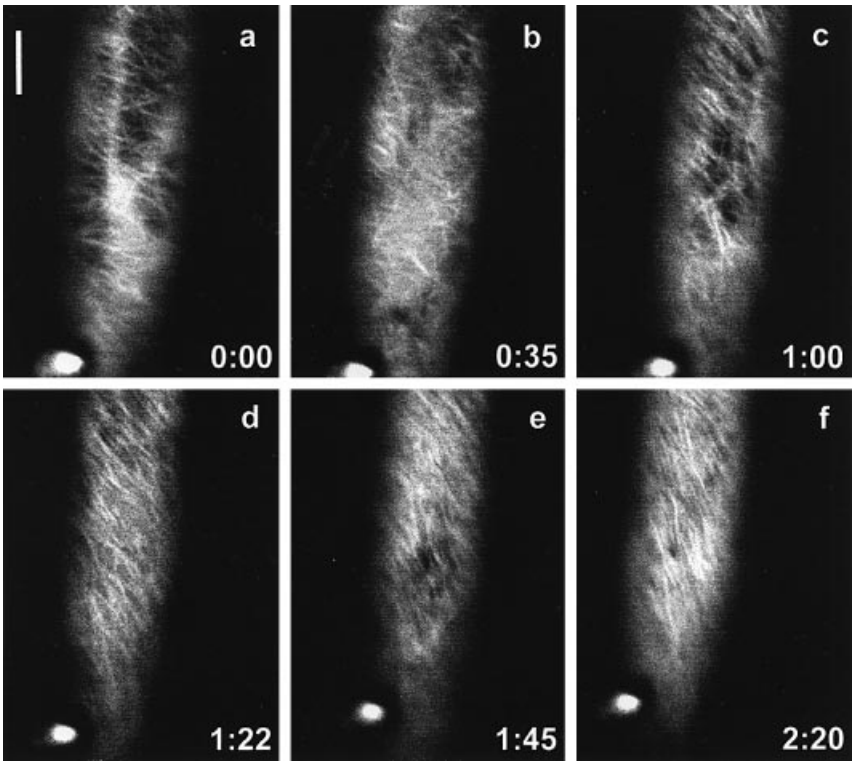

Figure 1. Gravitropically induced microtubule reorientation from transverse to longitudinal imaged *in vivo*.
Cortical microtubules under the outer wall of living maize coleoptile epidermal cells (upper flank) reorient from transverse to longitudinal in response to gravitropic stimulation. The same region of this cell was imaged at different time points (exact time point see lower right corner of each image) after injection of rhodamine-labelled tubulin. Bar = 5 μm.

Microtubule dynamics

To obtain better time resolution of the dynamic processes involved in gravity-triggered microtubule reorientation, images of *in vivo* labelled microtubules were recorded every 3–10 min (Figure 3). Changes in microtubule patterns were obvious despite the short interval between subsequent images (Figure 3, short arrows). As shown in Figure 3, some microtubules disappeared within minutes (short arrows) whereas others stayed at the same position for several minutes (long arrows), which is consistent with previous findings in *Nitella* cells (Wasteneys *et al.*, 1993). This dynamic nature of microtubules makes it difficult to identify a given individual microtubule (or microtubule bundle) in subsequent records of a microtubule array.

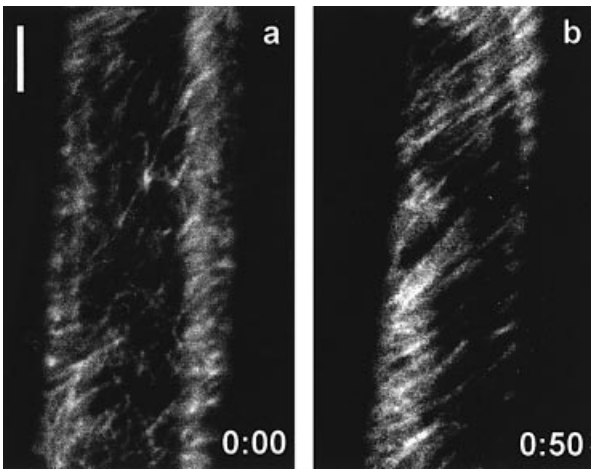

Figure 2. Randomly organized microtubules as a transitional state during microtubule reorientation.
As in Figure 1, randomly organized microtubules (15 min after the onset of gravitropic stimulation, a) initiated the reorientation process before they became co-aligned 50 min later (b). Bar = 5 μm.

Discussion

Mixed microtubule arrays: transitional states during reorientation

To follow microtubule reorientation in response to defined gravistimulation, we observed microinjected labelled tubulin in living cells. This is the first time that this has been reported for epidermal cells of intact maize coleoptiles. The microinjection technique allows microtubules to be observed in individual cells over time and should allow insight into the mechanism of microtubule reorientation. Two models were proposed that can explain microtubule reorientation: (1) Microtubule organization could be altered by movement or sliding of individual microtubules. (2) The directional reassembly model suggests selective

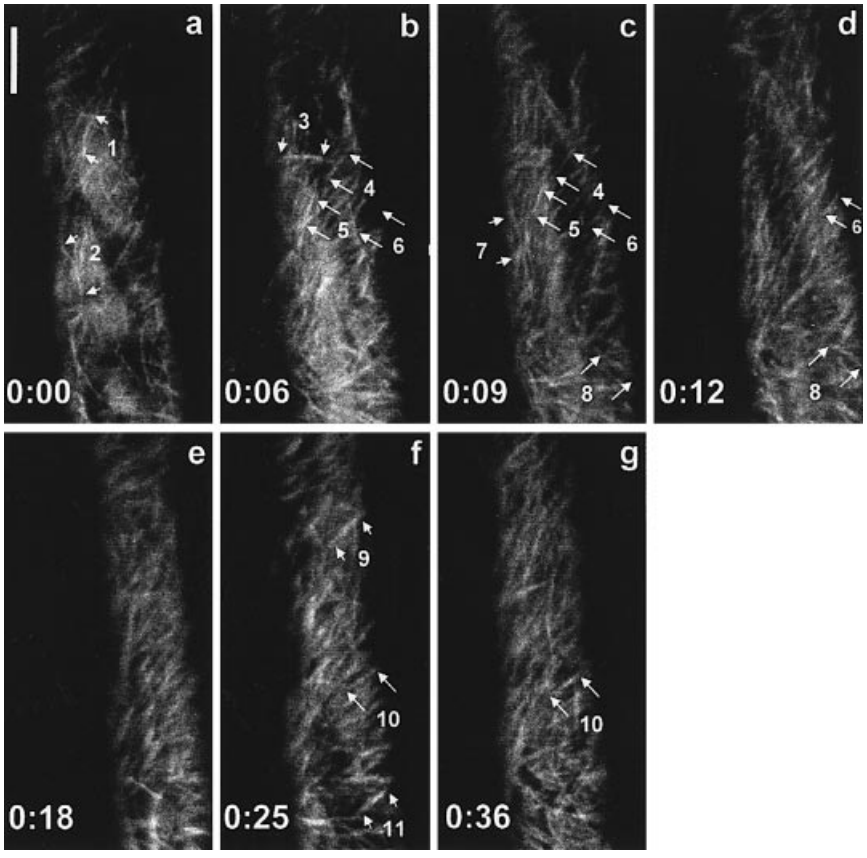

Figure 3. Microtubules exhibit differential dynamics during reorientation.
Cortical microtubules were imaged every 3–10 min (for exact time point see lower left corner of each image) after injection of rhodamine-labelled tubulin. Each image shows the same region of the cell. Short arrows indicate microtubules which disappear within only a few minutes, whereas microtubules that stay at the same position for up to 10 min ('pseudostable microtubules') are indicated by long arrows. Bar = 5 μm.

stabilization/disassembly of microtubules based on the high microtubule dynamics. In this case, the equilibrium between assembly and disassembly depends on the direction of a given microtubule. Thus, microtubules in the 'old' direction are selectively shrinking whereas microtubules in the 'new' direction are growing.

In several cases, we were able to observe the formation of randomly organized microtubule patterns as a transient state that became realigned into increasingly parallel arrays from about 40 min after the onset of gravitropic stimulation. In immunofluorescence studies of cell populations (e.g. Duckett and Lloyd, 1994), microtubule arrays are usually classified as either transverse or oblique or longitudinal, although a small fraction of arrays cannot be classified in this way and is described as random or mixed. From their microinjection studies on the reorientation of microtubules in pea epidermal cells, Yuan *et al*. (1994) suggested that these random arrays could be key transitional stages (see also Lloyd, 1994). We now show that this mechanism also applies to gravity-induced reorientation in maize epidermal cells, indicating the random microtubule arrays as a general feature of transition from transverse to longitudinal.

As shown in Figure 1(b–d) and Figure 2, these chaotic microtubule arrays become realigned which resulted in parallel, organized microtubules in an oblique orientation. In many cases, a smooth transition to steeper orientations was observed as soon as microtubules became clearly organized in parallel arrays (Figure 1d–f) confirming previous studies in pea epidermal cells (for review see Wymer and Lloyd, 1996). This realignment seems to involve a movement of the whole microtubule array that is probably independent from microtubule turnover since Wymer *et al*. (1996) have shown for BY-2 cells that microtubule arrays realign even faster when stabilized with taxol, a blocker of microtubule disassembly. Motor proteins that move along microtubules and structural microtubule-associated proteins (MAPs) that crosslink microtubules should be important for these sliding processes, and it is interesting that Chan, Rutten, Bush, Allan, Hussey and Lloyd (submitted) have recently described a kinesin-related protein on cortical microtubules.

As seen by imaging at a high time resolution (Figure 3), some microtubules can maintain the same position over several minutes (Figure 3: MT nos 4, 5, 6, 8, 10). This contrasts strikingly with the observed extremely fast incorporation of the labelled tubulin which is consistent with data on rapid microtubule turnover (Hush *et al*., 1994). Such microtubules could appear to be 'pseudostable' because tubulin is continuously treadmilling through them. Adjacent microfilaments or connection to the cell wall could hold them in one position. A transient detachment of microtubules from the wall would allow microtubules to gain a new orientation. Indeed, it has been shown recently for tobacco cells that microtubule organization can be disturbed by inhibiting cellulose synthesis (Fisher and Cyr, 1998), suggesting a feedback loop between cortical microtubules and cellulose microfibrils. This is supported by electron microscopy images that suggest connections between microtubules and cellulose fibres (for instance Giddings and Staehelin, 1988; Vesk *et al*., 1994). Attachment to the cell wall was also reported to stabilize microtubules, e.g. against cold (reviewed by Shibaoka, 1994).

Conclusion

We propose a microtubule reorientation mechanism that consists of the following steps: (1) a transitional stage in which parallel, organized microtubules lose their order due to the appearance of individual microtubules in random orientation. (2) Subsequently, a new order slowly emerges, starting with a few microtubules in a slightly oblique orientation. These oblique microtubules in the 'right' orientation could be the result of selective stabilization/disassembly (model B). (3) Next, a co-alignment process follows during which neighbouring microtubules adopt this oblique orientation (perhaps with the help of motor proteins or structural MAPs). (4) Eventually, the angle increases slowly with only smooth transitions in the microtubule orientation. The co-alignment process in (3), as well as the smooth transitions in the angle of microtubule arrays (4), could be caused by sliding of intact microtubules.

Experimental procedures

Plant material

Caryopses of maize (*Zea mays* L. cv. Percival; Asgrow, Bruchsal, Germany) were rinsed in tap water for 1 h and then sown on moist paper towels. The seedlings were cultivated at 25°C under continuous red light (0.2 W m^{-2}) for 3 days resulting in a predominantly transverse microtubule orientation. Microinjection and gravitropic stimulation were carried out under the red light used for cultivation. During microinjection, stray white light from the microscope bulb was confined to the utmost minimum. The green light used to activate rhodamine fluorescence is known not to induce microtubule reorientation (P. Nick, unpublished observations). For gravitropic stimulation, seedlings were positioned horizontally with the flat side of the coleoptile adjacent to the caryopsis facing upwards.

Preparation and microinjection of rhodamine–tubulin

Preparation and microinjection of rhodamine–tubulin was carried out according to Wymer *et al*. (1997). Prior to injection, coleoptiles were excised at the base, the primary leaves were removed and the coleoptiles mounted onto coverslips with medical adhesive (B-401, Factor II Incorp., Lakeside, AZ, USA). Coleoptiles were subsequently kept in a horizontal position corresponding to a gravitropic stimulus of 1 g with the flat side of the coleoptile facing upwards as described above. Rhodamine-conjugated

tubulin was injected into epidermal cells. During the whole procedure the coleoptiles were well supplied with water. Between 10 and 20 min after injection (time point 0) rhodamine-labelled microtubules underlying the outer wall were imaged using a Zeiss Universal microscope coupled to a Bio-Rad MRC-600 confocal laser scanning system. Further images of the same arrays were also taken at later time points. Rhodamine–tubulin was visualized using the 568 nm line of the Krypton-Argon laser.

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